Isolation of Messenger Ribonucleoproteins from HeLa Cells by Affinity Chromatography on Poly (U) Sepharose

Jean-Pierre Liautard *, Dagmar Tromm, and Kurt Köhler

Biologisches Institut der Universität Stuttgart

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 Messenger Ribonucleoproteins

Polysomes of HeLa cells were adsorbed on Poly(U)-Sepharose columns. This adsorption is probably due to poly(A) sequences at 3' terminus of the messenger. Stepwise elution initially removed ribosomal subunits thereafter mRNA and a set of proteins.

These proteins are identical with the main components of the polysomal messenger ribonucleoprotein particles described previously. Thus, this method allows their rapid and easy separation from ribosomal and other proteins.

Introduction

Messenger RNAs with poly (A) sequences at their 3'ends have been isolated successfully from various tissues by column chromatography using poly(U) or oligo(dT) covalently bound to matrices 1–4.

Lindberg 5 prepared polysomal mRNP particles by the same principle by passing cytoplasmic extracts through a column of oligo(dT) linked to cellulose. Since mRNPs are an integral part of the polysomal complexes, we have tried to fix whole polysomes to such a column, hoping that free 3'ends of mRNA would bind to the immobilized poly(U).

Preliminary experiments indicated that this could be accomplished. It was further possible to remove the main constituents of the bound polysomes by stepwise elution. We have characterized the eluted fractions and prove that mRNAs and their associated proteins can be obtained by this means.

Materials and Methods

Buffers

Buffer A: 10 mM Tris-HCl (pH 7.4); 10 mM KCl; 3 mM MgCl₂; 7 mM mercaptoethanol.
Buffer B: 10 mM Tris-HCl (pH 7.4); 500 mM KCl; 3 mM MgCl₂; 7 mM mercaptoethanol.
Buffer I (isotonic): 10 mM Tris-HCl (pH 7.4); 150 mM KCl; 3 mM MgCl₂; 7 mM mercaptoethanol.

Abbreviations: mRNP, messenger ribonucleoproteins; EDTA, ethylenediaminetetraacetic; DOC, deoxycholate; poly(U), polyuridylic acid; poly(A), polyadenylic acid.

* Laboratoire de Biochimie, Université des Sciences et Techniques du Languedoc, Place E. Bataillon, 34000 Montpellier, France.

Requests for reprints should be sent to Dr. K. Köhler, Biologisches Institut der Universität Stuttgart, Ulmer Strasse 227, D-7000 Stuttgart.

Buffer W (wash buffer): 10 mM Tris-HCl (pH 7.4); 500 mM KCl; 3 mM MgCl₂; 1 M urea; 7 mM mercaptoethanol.
Buffer E (elution buffer): 20 mM Tris-HCl (pH 9.0); 6 M urea; 7 mM mercaptoethanol.
Buffer S (containing sarkosyl): 150 mM KCl; 0.5% sarkosyl; 0.2% Na₂S.

Cell culture and fractionation

HeLa cells (clone S3) were grown in Eagle's medium complemented with 5% calf serum. Cellular RNAs were labeled with 0.2 µCi/ml [3H]uridine for 12 hours incubation. A ten times concentrated cell suspension was grown in the presence of 0.05 µg/ml actinomycin D for 20 min followed by the addition of [3H]uridine (1 µCi/ml) so that mRNA was preferentially labeled 6. After 3 hours incubation the cells were sedimented by centrifugation, resuspended and washed with buffer A, and homogenized. Nuclei and mitochondria were removed by centrifugation. The post-mitochondrial supernatant was layered on a 1.5 ml 30% sucrose cushion in buffer B and centrifuged in a SW 50.1 rotor at 25 000 rpm for 17 hours. The pellet was immediately dissolved in buffer I or stored at — 80°C.

Affinity chromatography

This procedure was carried out at 4°C. 1.5 g of poly(U)-Sepharose (obtained from Pharmacia) was allowed to swell in buffer E for 10 min, and then packed in a column of 1 cm diameter. The column was washed with 50 ml buffer E and equilibrated with 100 ml of buffer I. Samples of 8 mg of polysomes were prepared in 3 ml of buffer I and applied to the column. The column was washed with 50 ml buffer I, followed by 20 ml buffer W, and eluted with buffer E. The column was finally washed with buffer S, and stored in this buffer pro up to 1 week. Columns were used 3 times.
Protein electrophoresis

To 1 ml of the solution for analysis 40 μl of iodoacetamide and 5 μl mercaptoethanol and 200 μl of 50% TCA were added successively. Proteins were allowed to precipitate overnight at 4 °C, and the samples were centrifuged for 20 min at 3000 g at 4 °C. The pellets were washed in ethanol/ether (1/1), then in ether and dried at room temperature. Proteins were dissolved in appropriate amounts of buffer (20 mM Tris-HCl (pH 7.4), 21 mM mercaptoethanol, 2% SDS) and heated in boiling water for 2 min. 10% acrylamide gels with a bisacrylamide/acrylamide ratio of 0.013 were made according to Favre and Laemmli. Acrylamide solution was poured in 0.6 cm diameter glass tubes and allowed to polymerize overnight. 1 cm of 3% acrylamide (bisacrylamide/acrylamide = 0.05) was deposited on the 8 cm long separating gel. Electrophoresis was run at room temperature at 3 mA/gel. The proteins were stained with Coomassie Brilliant Blue and molecular weights were estimated by comparison with standard proteins.

Centrifugation in CsCl

To a 100 μl sample for analysis 10 μl of 30% glutaraldehyde neutralised with 1 M Na₂CO₃ were added. Fixation took place at 4 °C for 5 min, then the resulting solution was layered on to pre-formed CsCl gradients and immediately centrifuged.

RNA analysis

Two volumes of chloroform/phenol were added to one volume of RNA-containing sample. RNA was extracted at room temperature according to Perry et al. The RNA was analysed on 2.2% acrylamide gels containing 0.5% agarose as described by Thiollais et al.

Results and Discussion

A set of preliminary experiments indicated that polysomes have a high affinity for poly(U)-Sepharose. This affinity is largely due to binding to poly(U) although other less specific binding cannot be excluded (Table I). From a variety of eluants a schedule of elution was established. The most effective procedure is as follows: When the total ribosome/polysome fraction from a sucrose gradient is loaded on a poly(U)-Sepharose column, the bulk of it passes through unadsorbed. However, as much as 20% of the original fraction remains on the column and is not removed by extensive washing with isotonic or hypotonic buffer; buffered 0.5 M KCl releases only some proteins. When, however,

<table>
<thead>
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<th>Column matrix</th>
<th>&quot;unbound&quot;</th>
<th>&quot;bound&quot;</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(U)-Sepharose</td>
<td>28% ±3%</td>
<td>72% ±3%</td>
<td>8</td>
</tr>
<tr>
<td>poly(A)-Sepharose</td>
<td>71%</td>
<td>29%</td>
<td>3</td>
</tr>
<tr>
<td>mere Sepharose</td>
<td>74%</td>
<td>26%</td>
<td>3</td>
</tr>
</tbody>
</table>

1 M urea in 0.5 M KCl buffer is added to the column, ribosomal RNA and ribosomal proteins are quantitatively eluted. In a further step the column is flushed with buffered 6 M urea (Buffer E); which removes more proteins and heterogenous RNA. The remainder of the total RNA (about 2%) can be detached by means of detergents.

For a closer analysis of the fractions eluted with the various buffers, HeLa cells were allowed to grow in the presence of [³H]uridine and actinomycin D under conditions where predominantly mRNA and 5S RNA are labeled. When a polysomal/ribosomal fraction from such cells is passed through the column (step 1) 85% of the radioactive RNA is retained; approximately 15% is not adsorbed and constitutes fraction A in Fig. 1. The

Fig. 1. Fractionation of polysomes on a poly(U)-Sepharose column. Approximately, 5×10⁸ cells were grown in the presence of [³H]uridine and actinomycin D in order to label mRNA; poly(U)-Sepharose column chromatography was performed as described in Materials and Methods. Fractions of eluate were collected and precipitated with 10% TCA at 4 °C. Precipitates were adsorbed on glass fibre filters, rinsed, and dried thoroughly. Radioactivity was determined in a Packard Scintillation counter.
following wash (step 2) with buffered 0.5 M KCl and 1 M urea removes approximately 15% and gives fraction B. From these two fractions RNA was extracted. They contain 18S, 28S and 5S RNA as revealed by polyacrylamide gel electrophoresis (Fig. 2). The column was then rinsed with buffered 6 M urea (step 3) yielding fraction C and finally (step 4) treated with sarkosyl to remove all tenaciously bound radioactivity (fraction D). As shown in Fig. 2 fraction C contains heterogeneous RNA.

We have further characterized the protein composition of the four fractions. Fraction A and B contain the spectrum of ribosomal proteins. Some apparently nonribosomal proteins with molecular weights above 55 000 daltons are also present; they probably represent factors of the “salt wash” (Fig. 3 a and b). Fractions C and D reveal a set of protein bands with two major proteins of molecular weights of 51 000 and 78 000, of the same order as those found in mRNPs. There are, also several minor bands (above 50 000 daltons) which have been previously reported by many groups12–14 (Fig. 3 c and d). Other minor bands with lower molecular weights may represent contamination with ribosomal proteins as suggested by the split gel electrophoresis procedure shown in Fig. 4. The two major proteins in fraction C do not coincide with any ribosomal protein in fraction B or proteins of fraction A.

It was of interest to know if the material eluted from the column is derived from defined, pre-existing particulate units. We have, therefore, analysed
the eluates by sucrose gradient centrifugation. Fraction A contains essentially ribosomes and ribosomal dimers or disomes (Fig. 5 a). Fraction B contains two particles with sedimentation rates of ribosomal subunits (Fig. 5 b). The analysis of fraction C reveals a heterogeneous group of particles with sedimentation rates below 60S (Fig. 5 c). However, since fraction C contains 6 M urea, it seems unlikely that the eluate consists of unaltered and integral particles; yet, since they appear upon gradient centrifugation, they have apparently been re-formed in the gradient. In order to test this, the eluate C was analysed by CsCl density gradient analysis after fixation with glutaraldehyde. By this procedure no ribonucleoprotein particles could be detected in fraction C (Fig. 6 a), instead RNA was found (Fig. 6, fraction 1 to 3). If fraction C was first dialysed against buffer I, aggregates re-form from heterogenous mRNA and proteins, yielding particles with a density in CsCl of 1.5 g cm\(^{-3}\) (Fig. 6 b). The density of these particles is equal to the density of “salt washed” ps-mRNPs, as found earlier in our laboratory\(^\text{15}\).

When in step 3 of the elution schedule 6 M urea was replaced by a buffer containing DOC, a fraction C' containing several proteins of was eluted. As seen from Fig. 7 the spectrum of proteins resembles the one obtained from ps-mRNPs, although only on of the two major proteins (75 000 daltons) was detected, together with several minor ones. The second major protein (50 000 daltons) was eluted only by the following ionic detergent treatment in step 4. This is in agreement with previous results from this laboratory, indicating that DOC removes protein from ps-mRMP, rendering them incapable of reassociating with ribosomes\(^\text{12}\); this is most likely due to the removal of the 75 000 daltons protein. EDTA, when used in step 3 (compare: Fig. 1), liberates no protein from the column\(^\text{16–18}\).
The results reported above indicate that in an immobilized state polysomal particles and their constituents behave as they do in solution. The immobilized state is most likely due to a binding of poly(A) sequences to poly(U). The choice of the right sequence of eluants facilitates the stepwise isolation of constituents of the functional polysomal particles including proteins of the ps-mRMP.

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